amplified by PCR using mouse brain cDNA library as a template with the following primer set: 5′-ATG GCG CAG GAG TCT TGC CA-3′ and 5′-TCA GGA GAG GCT GCT GTC CTT TTT GC-3′. The PCR product was subcloned into pCR II-TOPO (Invitrogen). pcDNA3.1-Myc/HisPtpcd1 was then generated by subcloning of the KpnI/NotI fragment into pcDNA3.1Myc/His vector. The KpnI/NotI fragment was amplified by PCR using pCRII-TOPOPtpcd1 as a template with a set of primers; 5′-TTT GAA TTC GCC ACC ATG TCG TCC GGG GCC AAG GAG-3′and 5′-AAA TCT AGA GGG CAG AGG GGT CCC GTT-3′. pcDNA3.1Plk1-HA was generated by subcloning of EcoRI/NotI fragment from pET23d-Plk1 (kind gift from Dr. Nishida) into pcDNA3.1HA.

Cell culture. HeLa and U2OS cells were cultured and maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum as described previously [19]. Nocodazole and hydroxyurea were purchased from Sigma.

Knockdown experiments. Plasmid transfections were performed using FuGENE6 transfection reagent (Roch) according to manufacturer's instruction. Stealth siRNAs for Plk1 (Invitrogen) or control (Invitrogen) were transfected using lipofectamin 2000 (Invitrogen).

Immunoblotting. Antibodies used in this study were anti-c-myc (sc-789; Santa Cruz, sc-40; Santa Cruz), anti-pTyr-Cdk1 (9111; Cell signaling), anti-cyclin B1 (GNS1; Santa Cruz), anti-Plk1 (35-200; upstate), anti-HA (12CA5; Roch), anti-γ-tubulin (T3559; Sigma) and anti-α-tubulin (Sigma). Whole cell extracts were prepared as described previously [20] and were subjected to immunoblotting. For IP-immunoblotting, U2OS cells were cotransfected with Plk1-HA, along with Ptpcd1-myc or empty pcDNA3.1. Cells were harvested in RIPA buffer (10 mM Tris–HCl, pH7.5, 150 mM NaCl, 0.5% Triton X-100, 1% sodium deoxycholate and complete protease inhibitor tablets (1/10 ml) (Roch). The lysates (200 μg) were incubated with anti-myc antibodies (5 μg) or mouse normal IgG as a control at 4 °C for 1 h and then precipitated with 20 μl of protein G beads. The resultant precipitates were separated by SDS–PAGE and then analyzed by immunoblotting.

Microtubule regrowth assay. Cells on coverslips were treated with 4 mM nocodazole for 30 min at 37 °C, followed by replacement with normal medium. Cells were fixed at −20 °C with methanol and immunostained for immunofluorescence microscopy was performed as described previously [21].

Immunofluorescence analysis. Secondary antibodies for immunofluorescence microscopy were Cy3 (Jackson immunoresearch), Alexa 594 and Alexa 488 (Molecular probes). Cells on coverslips were fixed in 4% PFA for 10 min at RT and then permeabilized with 0.25% Triton X-100 in PBS. Blocking was done in 5% normal goat serum (Convac) containing 1% Triton X-100. Fixed cells were incubated with antibodies in blocking for 1 h at RT. DNA was counterstained with DAPI (2 mg/ml). For centrosomal staining, cells were fixed with methanol at −20 °C for 10 min.

Results and discussion

Ptpcd 1 is a centrosomal phosphatase that regulates centrosomal duplication during S phase

Immotile primary cilium is a centriole-based organelle that consists of microtubule pairs located at the plasma membrane [22]. The fact that a cilium is differentiated from centrosomes after mitosis has suggested that the function and structures of cilia are regulated by similar mechanisms to those of centrosomes [23]. Indeed, Nek2 and Aurora A kinases are involved in the regulation of both centrosomes and cilia functions [24]. Ptpcd 1 was originally identified as a dual specificity protein phosphatase highly expressed in mouse cilia [25]. We therefore speculated that Ptpcd 1 might be involved in the regulation of centrosomal function. Ptpcd 1 encodes a 721 amino acid protein with a predicated molecular weight of 82 kDa. Sequence analysis of Ptpcd1 revealed the significant homology with yeast Cdc14p with coiled coil domain at its carboxyl terminal, RXXL motif (a putative APC/C binding motif), several nuclear localization signals (Suppl. Fig. 1A), and a putative nuclear export signal (Suppl. Fig. 1B). To examine whether Ptpcd1 functions at centrosomes, Ptpcd1-myc was transfected into HeLa cells. In interphase cells, signals corresponding to Ptpcd1 were detected at both cytoplasm and nucleus with one or two closely spaced dots. Importantly, these dot signals were localized adjacent to, but not overlapped with, the signals from γ-tubulin, a centrosomal protein, raising the possibility of its centriolar localization (Fig. 1A). This centrosomal enrichment of Ptpcd1 was observed from interphase to anaphase, whereas it was hardly detected at telophase where most of the immunoreactivity was detected at midbody. This spatio-temporal localization in relation to cell cycle conveyed that Ptpcd1 might be a novel centrosomal-related phosphatase.

In certain transformed cells, such as U2OS cells, prolonged S phase arrest by hydroxyurea (HU) causes uncoupled centrosomal duplication from cell cycle with multiple rounds of centriole duplication in the absence of DNA replication and mitotic division (Fig. 1B, upper panel) [15]. In order to examine whether Ptpcd-1 plays a role in the regulation of centriole duplication, Ptpcd1 were overexpressed into U2OS cells in the presence of 4 mM HU for 72 h. Ptpcd 1 expression inhibited HU-induced centriole over duplication (Fig. 1B, lower panel). Majority of control U2OS cells possessed more than single centrosome when cells were treated with HU. In contrast, 60% of Ptpcd 1 expressing cells possessed single centrosome (Fig. 1C). These results clearly indicated that overexpression of Ptpcd1 suppressed uncoupled centrosomal duplication during S phase. Given that Cdk activity regulates centrosomal duplication during S phase, we asked whether Ptpcd1 affect inhibitory phosphorylation of Cdc2 at Y15, which is regulated at centrosomes during S to G2 phase. Cdc2 phosphorylation at Y15 slightly decreased, whereas expression of cyclin B1 was not affected by Ptpcd1 expression (Fig. 1D).

Ptpcd1 associates with Plk1 and is involved in the regulation of mitotic progression

Plk1 regulates centrosomal functions at multiple levels, such as centrosomal stabilization, nucleation, duplication, microtubule stabilization, and cytokinesis [9–13]. Therefore, Ptpcd1 might be involved in Plk1-dependent regulation of centrosome functions. To examine this possibility, we first asked whether Ptpcd1 physically interact with Plk1. Plk1-HA and Ptpcd1-myc were co-transfected into HeLa cells and the cell extracts were immunoprecipitated with anti-myc antibodies. Plk1-HA as well as Ptpcd1-myc was readily detected in the myc-immunoprecipitates, indicating that Plk1-HA was capable of forming a complex with Ptpcd1-myc (Fig. 2A). Immunocytochemical analysis revealed colocalization of ectopically expressed Ptpcd1-myc with endogenous Plk1 (Fig. 2B). Intriguingly, Ptpcd1-myc appeared to accumulate and colocalize with Plk1 at mid-body in telophase cells. These results suggested that Ptpcd1 might also function in cytokinesis as Plk1 did [1]. To address this question, we examined the effect of Ptpcd1 overexpression on mitotic progression. Aberrant cytokinesis in the form of fused or intercellular α-tubulin bridges was apparent in cells expressing Ptpcd1 (Fig. 3A and C). Hence, an increase in the number of cells with multiple nuclei was observed when Ptpcd1 was overexpressed (Fig. 3B and C). These results suggested that Ptpcd1 as well as Plk1 as reported played an important role in cytokinesis.

In addition to cytokinesis, Plk1 was reported to stabilize microtubule organization [10,12]. Therefore, we then asked if Ptpcd1 expression affect the microtubule stabilization. HeLa cells were transfected with pcDNA3.1Ptpcd1-myc and treated with nocodaz-

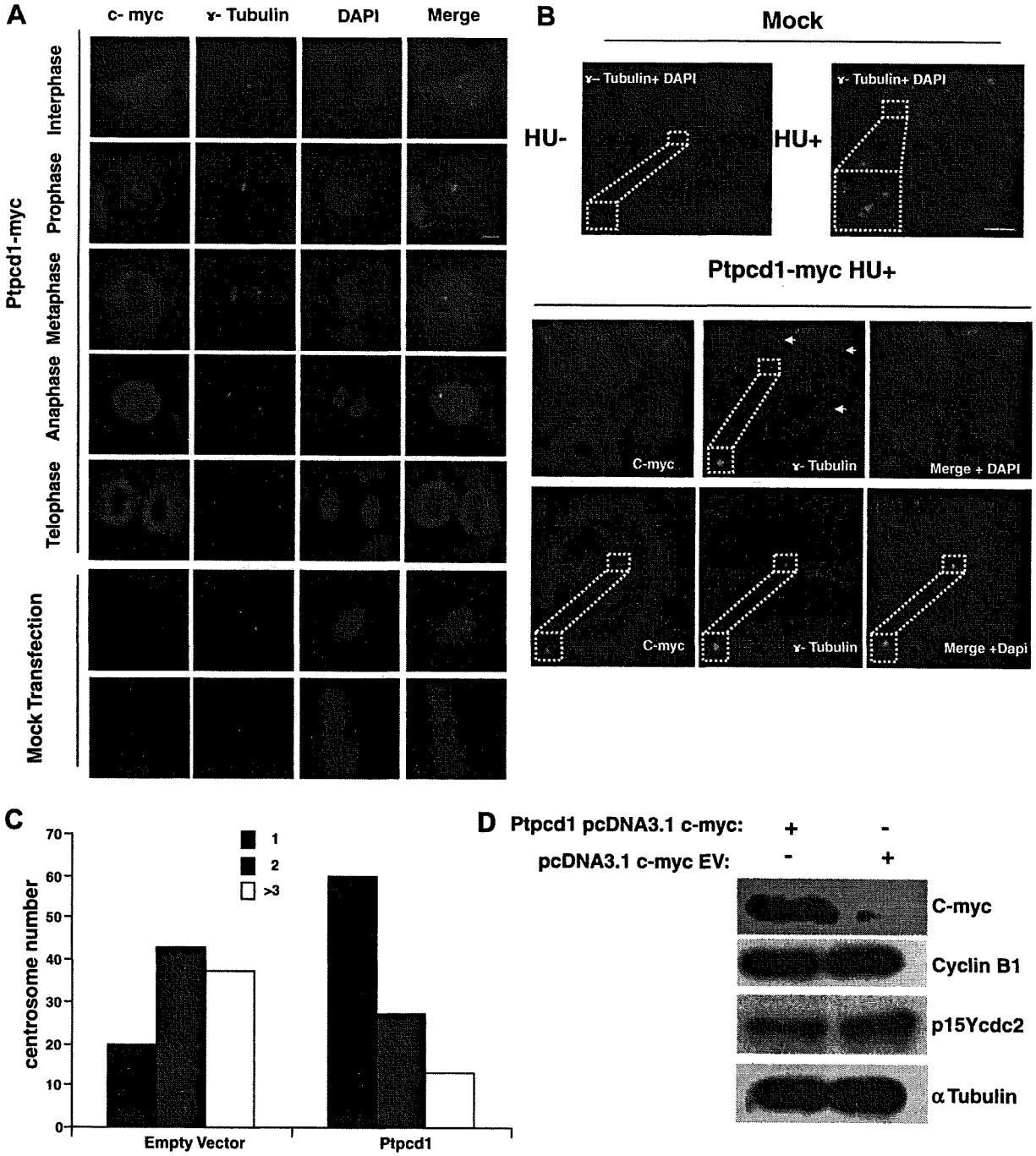

Fig. 1. Ptpcd1 localized to centrosomes and its overexpression prevents unscheduled centriole duplication. (A) Either Ptpcd1-myc or empty pcDNA3.1 as a negative control was transfected into HeLa cells. Cells were fixed with methanol and co-immunostained with γ-tubulin (green) and c-myc (red). DNA was counterstained with DAPI (blue). Scale bars; 5 μm. (B) Overexpression of Ptpcd1 prevents unscheduled centriole duplication. U2OS cells were transfected with either empty vector the presence or absence of HU (4 mM). (upper panels) or Ptpcd1-myc (lower panels) in the presence of HU (4 mM). Cells were then fixed and immunostained with anti-γ-tubulin antibodies (green) and c-myc (red). Arrows point at centrosomes, enlarged insets showed centrosomes. Scale bars; 5 μm. (C) The number of cells with 1, 2, or more than 2 centrosomes from (B) was counted and shown as a percentage of total cells. (D) Immunoblotting analysis. Whole cell extracts from (B) were subjected to immunoblotting using the indicate antibodies. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

ole, a microtubule depolymerizing agent. Overexpression of Ptpcd1-myc significantly stabilized microtubule organization when it was evaluated by staining with α-tubulin (Fig. 3D). Interestingly, it also enhanced microtubule regrowth after nocodazole washout. Thus, the effect of Ptpcd1-myc expression on microtubules stabilization was similar to that of Plk1 expression, further suggesting that Ptpcd1 functioned in Plk1-regulatory networks. In addition, these results also showed that the centrosomal localization of Ptpcd1 was independent of microtubules nucleation because centrosomal signals of Ptpcd1 could still be detected after microtubules depolymerization with nocodazole.

Overexpression of Ptpcd1 is capable of complementing prometaphase arrest in Plk1 depleted cells

We finally determined the genetic orientation between Plk1 and Ptpcd1. Endogenous Plk1 was depleted by the transfection of the specific siRNAs (D-1 and D-2) for Plk1. Immunoblotting using

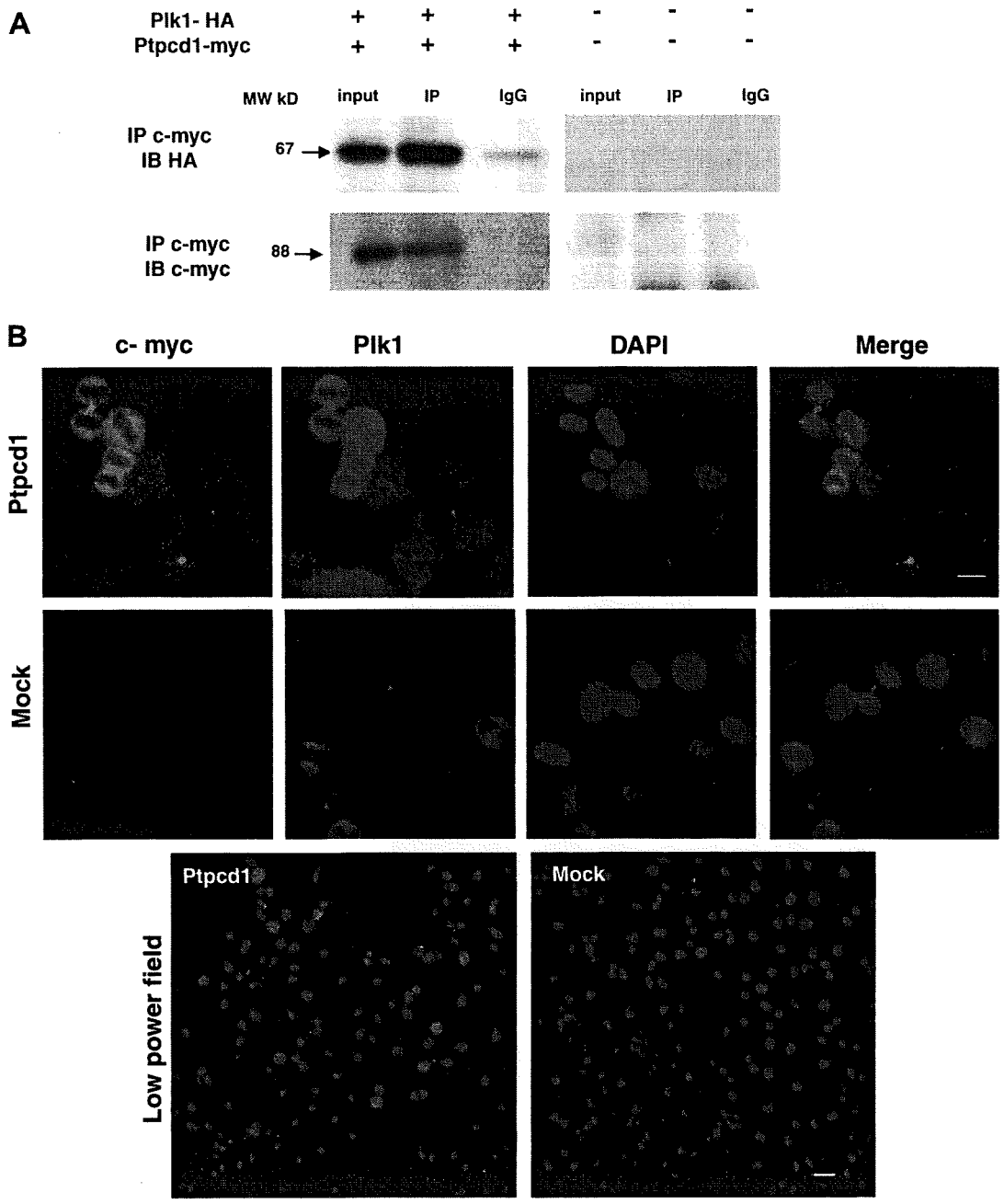

Fig. 2. Ptpcd1 interacted and colocalized with Plk1. U2OS cells were cotransfected with Plk1-HA and either Ptpcd1-myc or empty vectors (mock). Cells were harvested, and lysates were immunoprecipitated with anti-myc antibodies. Immunoprecipitates were subjected to immunoblotting using the indicated antibodies. (B) Colocalization of Ptpcd1 with Plk1. U2OS cells were transfected with Ptpcd1-myc and then stained with anti-myc antibodies (green) and anti-Plk1 antibodies (red, upper panels). DNA was counterstained with DAPI. Lower panels were low power fields of the upper images. Scale bars; 5 μm. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

anti-Plk1 antibodies revealed that transfection of D-1 and D-2 resulted in a significant reduction in Plk1 protein, whereas that of control siRNA did not (Fig. 4A). Plk1 depletion results in prometaphase/metaphase arrest [1], presumably due to impaired centrosomal separation, centrosome fragmentation, and microtubules destabilization, which consequently activate spindle checkpoints. Prolonged prometaphase arrest in Plk1 depleted cells ultimately appeared to induce apoptosis (Fig. 4B and Suppl. Fig. 2). Intriguingly, ectopic expression of Ptpcd1 in Plk1 depleted cells rescued the prometaphase/metaphase arrest and stabilized microtubules (Suppl. Fig. 2), but resulted in aberrant cytokinesis as was observed in Ptpcd1 expressing cells (Fig. 3C). These results indicated that Ptpcd1 at least in part functioned downstream of Plk1. Consistent with this notion, Ptpcd1 possessed four consensus serine residues for a Plk1 phosphorylation site (Fig. 4C) [26]. In this regard, Cdc5, a yeast homolog of Plks, regulated Cdc14p phosphorylation and its subcellular localization to ensure mitotic exit [14,27].

In summary, based on our observation in this study, it is possible that Ptpcd1 together with Plk1 may regulate the centriole duplication cycle as well as cytokinesis by modulating the phosphorylation status of some proteins, suggesting the conserved mechanism from yeast to mammalian by which Cdc5–Cdc14 axis regulates mitotic exit [1,14,27]. A similar counterbalance of kinase and phosphatase activities was also proposed in Nek2 and PP1α

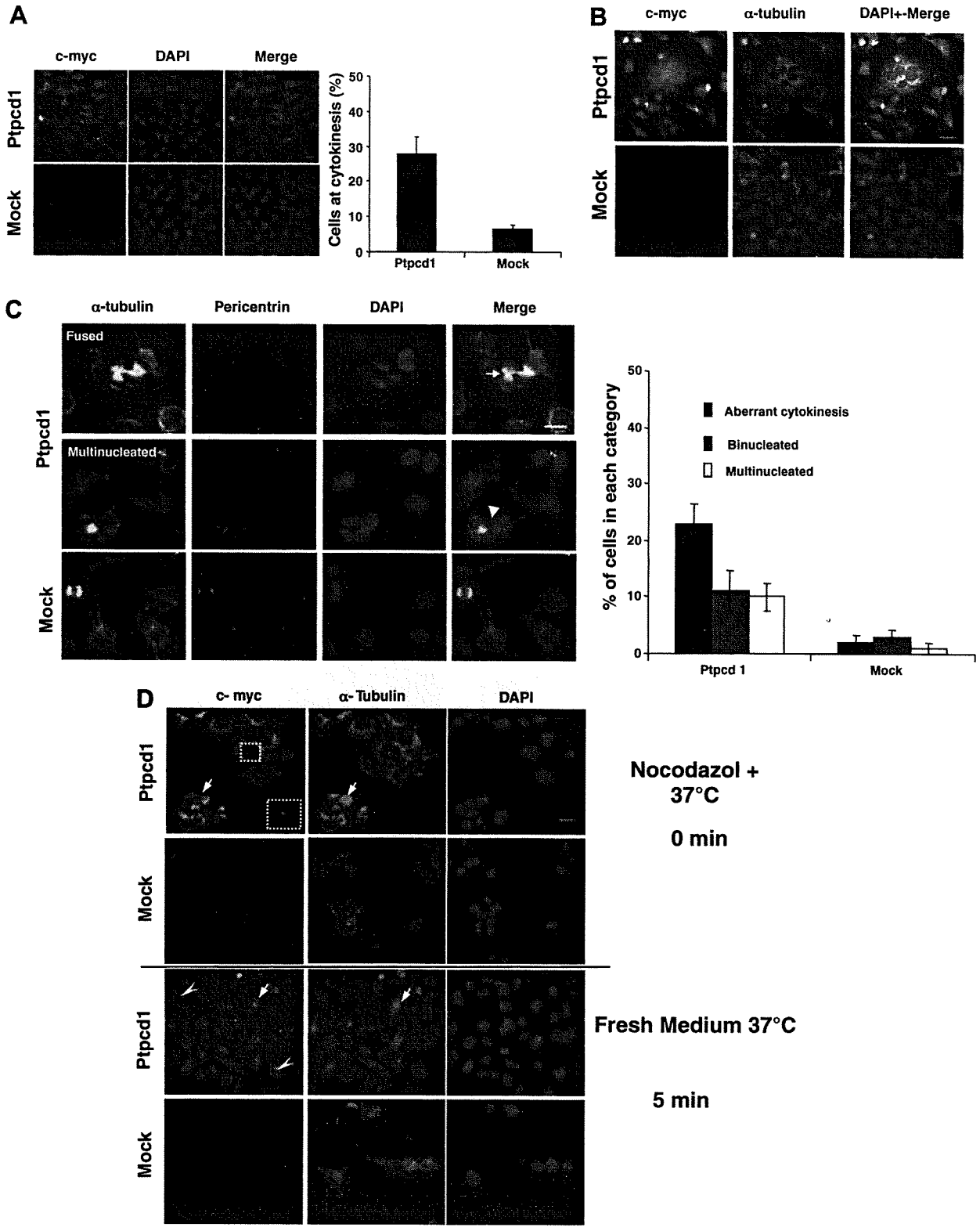

Fig. 3. Overexpression of Ptpcd1 resulted in aberrant cytokinesis and microtubules stabilization. (A) HeLa cells were transfected with either Ptpcd1-myc or empty vector (mock). Cells were immunostained with anti-myc (green) and DNA was counterstained with DAPI (blue). Scale bars; 5 μm (left panel). Cells with cytokinesis were counted and the data were presented as a percentage of total cells ($n > 200$) and means ± SD from three independent experiments (right panel). (B) HeLa cells were transfected as in (A) and stained with the indicated antibodies. DNA was counterstained with DAPI. (C) HeLa cells were transfected as in (B) and immunostained with anti-tubulin (red) and pericentrin (green). DNA was counterstained with DAPI. White arrow indicated persistent midbody structure and arrowhead pointed at multinucleated cells (left panels). Scale bars; 5 μm. Cells with aberrant cytokinesis, binucleated, and multinucleated cells from (left panels) were counted and the data were presented as a percentage of total cells ($n > 100$) and means ± SD from three independent experiments. (D) Microtubule regrowth assay. HeLa cells were transfected with either pcDNA3.1Ptpcd1-myc or empty vector. Cells were then treated with nocodazole as in Materials and Methods, and stained with anti-myc (green) and anti-α-tubulin (red) antibodies (upper panels). Cells were further cultured with the medium in the absence of nocodazole for 5 min and then stained as described above (lower panels). White arrow indicated stabilized α-tubulin bundles by Ptpcd1 and arrow head indicated newly growing microtubules. Insets show centrosomal Ptpcd1, Scale bars; 5 μm. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

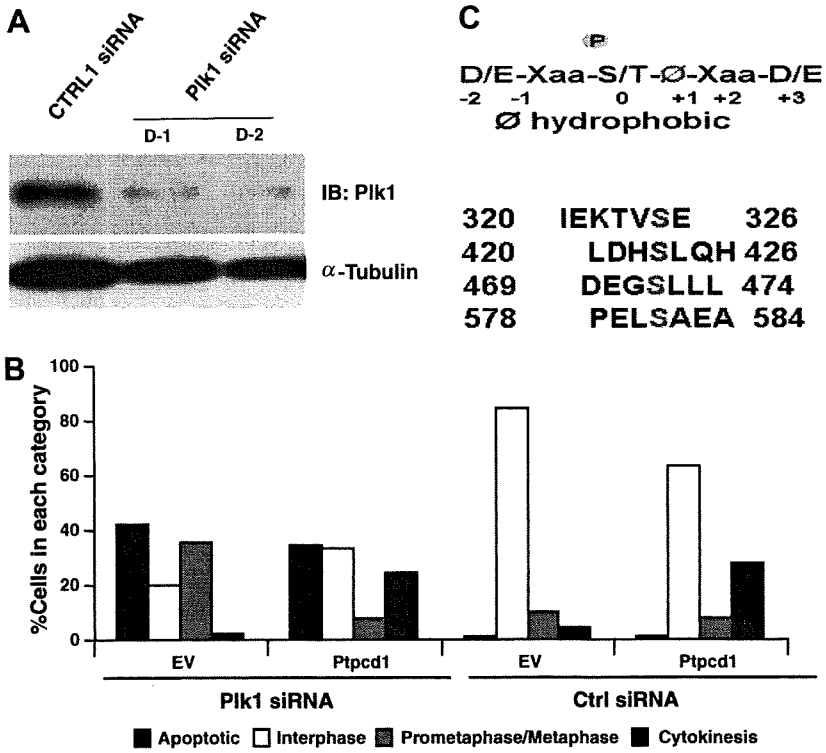

Fig. 4. Overexpression of Ptpcd1 rescued prometaphase/metaphase arrest observed in Plk1 depleted cells. (A) Plk1 depletion by transfection of its specific siRNAs. U2OS cells were transfected with the indicated siRNAs and the lysates were subjected to immunoblotting using the indicated antibodies. (B) U2OS cells were transfected with Ptpcd1 or empty vector (EV) as a negative control together with either Plk1 siRNA or control siRNA as indicated. The transfected cells were then fixed and immunostained stained with c-myc and α-tubulin. Cells in interphase, prometa/metaphase, cells with aberrant cytokinesis and/or underwent apoptosis were scored and the results were presented as a percentage of total cells ($n > 200$). (C) Schematic presentation of the predicted plk1 consensus phosphorylation site inside Ptpcd1 sequence. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

that governed centrosome splitting [4]. Although centrosomal and mitotic functions of mammalian Cdc14B had been described [15,17], Cdc14B deficient cells were viable and lacked apparent defects in chromosome segregation and cytokinesis [8], suggesting that alternative phosphatase(s) is being capable of complementing the mitotic functions of Cdc14B. Taken together with the fact that Ptpcd1 shared some sequence homology with Cdc14B, our results proposed that Ptpcd 1 might act as a functional isozyme to Cdc14B.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.01.113.

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